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Vaccinia virus induces apoptosis of infected macrophages

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Vaccinia virus (VV) infects a broad range of host cells, and while it usually causes their lysis (i.e. necrosis), the nature of the cell-death phenomenon is not well understood. In this study, we show that VV induces apoptosis of cells of the murine macrophage line J774.G8, as revealed by morphological signs, DNA ladder formation, changes of mitochondrial membrane potential and annexin-V positivity. Apoptosis occurred in both untreated and IFN-γ-pretreated macrophages, and could not be inhibited by aminoguanidine, a relatively specific inhibitor of inducible nitric oxide synthase. Inhibition of VV DNA synthesis and late gene expression by cytosine arabinoside also did not prevent apoptosis, while heat- or psoralen/UV-inactivated VV did not cause any apoptosis. Thus, VV early gene expression seems to be required for induction of apoptosis. At the cellular level, infection with VV induced a decrease in the levels of Bcl-x_L, an anti-apoptotic member of the Bcl-2 family. The importance of loss of Bcl-x_L was demonstrated by prevention of VV-mediated apoptosis on expression of Bcl-2, a functional homologue of Bcl-x_L. Our findings provide evidence that induction of apoptosis by VV in macrophages requires virus early gene expression, does not involve nitric oxide, induces a decrease in mitochondrial membrane potential and is associated with altered levels of Bcl-x_L.

Introduction

The outcome of virus infection of a host cell depends on many features of both the virus and the cell. Among various results of these virus–cell interactions, special attention has been paid to the self-destruction of the host cell by programmed cell death.

Programmed cell death, or apoptosis, is a frequent result of virus infection. As part of the host-cell defence mechanisms, it may reduce virus growth as well as its spread and dissemination within the organism. However, viruses modulate apoptosis in both directions: they block or delay apoptosis by specific virus-encoded factors in order to get sufficient virus progeny produced, or they use it as a strategy to get released from the cell. Sometimes, the host cell inhibits the virus-induced apoptosis (Teodoro & Branton, 1997; Barry & McFadden, 1998; Roulston *et al.*, 1999; Fujimoto *et al.*, 2000).

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Poxviruses have a special ability to survive and replicate within a host organism, despite a strong immune response. This ability is due to the expression of various virus-encoded genes that modulate or counteract the host response at both extracellular and intracellular levels (Buller & Palumbo, 1991; Haig, 1998). Poxviruses usually cause lysis (i.e. necrosis) of the infected cell, and they encode several factors opposing apoptosis directly or indirectly: factors that inhibit cytokine processing and proteolytic activation of caspases, soluble receptors neutralizing the effects of various cytokines, factors that inactivate IFN-inducible antiviral enzyme activities, and analogues of growth factors and hormones (Gagliardini *et al.*, 1994; Smith *et al.*, 1997, 1999; Bird, 1998; Nash *et al.*, 1999; Alcamí & Koszinowski, 2000; Everett *et al.*, 2000). Reports of apoptosis caused by poxviruses are rather rare, except those describing apoptosis induced by mutants deficient in some apoptosis-preventing factor (Kibler *et al.*, 1997) or by a recombinant protein expressed using vaccinia virus (VV) as a vector (Ronen *et al.*, 1996; Gil *et al.*, 1999; Timiryasova *et al.*, 1999). The examples of poxvirus-induced apoptosis include canarypoxvirus-infected primate dendritic cells (Ignatius *et al.*, 2000) and rabbitpox virus-infected pig kidney LLC-PK1 cells (Macen *et al.*, 1998). VV infection has also been observed to cause apoptosis in certain cells. VV induced apoptosis of Chinese hamster ovary (CHO) cells after virion binding to the

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cell surface, supposedly with no further requirements for VV early genes expression (Ramsey-Ewing & Moss, 1998). In contrast, VV early proteins were expressed in an immature B lymphocyte cell line WEHI-231 in which the virus caused apoptosis but did not replicate (Baixeras *et al*., 1998). Similarly, VV caused apoptosis of human dendritic cells with only VV early proteins being expressed (Engelmayer *et al*., 1999). Additionally, VV infection in mice was shown to lead to a lymphopenia, which has been ascribed to programmed cell death (Gonzalo *et al*., 1994).

Phagocytic cells of the monocyte/macrophage lineage play a central role in virus–host interactions. Their responses to VV infection have been studied in several species. In rabbit blood monocytes and macrophages, VV replication was found to be slow and the virus matured asynchronously (Jelinkova *et al*., 1975; McLaren *et al*., 1976). In mouse peritoneal macrophages, the VV replication cycle was found to be abortive with only early VV proteins expressed, neither VV DNA synthesis nor VV late proteins detected, and with no assembly of progeny virions (Natuk & Holowczak, 1985). Similarly, a study of the *in vitro* interactions between VV and monocyte-derived human dendritic cells revealed that only early virus-encoded proteins were expressed, while viral DNA synthesis and virus late protein expression did not occur (Drillien *et al*., 2000). In contrast, in activated rabbit peritoneal macrophages, a block in a late step of the virus replication cycle, after DNA synthesis, caused abortive replication of VV (Buchmeier *et al*., 1979). However, there have been no reports of monocyte or macrophage apoptosis induced by wild-type VV.

Cytotoxic and apoptosis-inducing properties of macrophages, as well as many of their antimicrobial and antiviral effects, are dependent on the activation of macrophages by IFN-γ and on induction of nitric oxide (NO) formation (Stuehr & Nathan, 1989; Nathan & Hibs, 1991; Nathan, 1992; Sarih *et al*., 1993; Reiss & Komatsu, 1998). Viruses themselves can be considered poor inducers of inducible NO synthase (iNOS) expression and NO production, but iNOS expression can be induced by other stimuli concomitantly with virus infection. iNOS expression appears to be mediated through induction of cytokines in a variety of experimental infections with viruses in rats and mice (Akaike *et al*., 1998), including neuroviruses (Zheng *et al*., 1993), pneumotropic viruses (Akaike *et al*., 1996) and cardiotropic viruses (Mikami *et al*., 1996). However, iNOS has also been reported to be induced directly by a virus structural component, a viral envelope glycoprotein of human immunodeficiency virus type 1, gp120 (Dawson *et al*., 1993).

Alteration of host-cell protein synthesis caused by viruses with a cytoplasmic site of replication, like VV, influence viability of the cell. During VV infection, many aspects of cellular processes are perturbed: nuclear DNA and RNA synthesis are inhibited (Kit & Dubbs, 1962; Becker & Joklik, 1964), the overall amount of protein synthesis is reduced (Shatkin, 1963) and viral polypeptides are exclusively synthesized (Esteban & Metz, 1973). This selective inhibition of host-cell protein synthesis (shut-off phenomenon) is thought to be mediated by VV virion-associated protein kinase (Buendia *et al*., 1987), as well as by virus-induced, untranslated, polyadenylated short RNA sequences (POLADS; Su & Bablanian, 1990; Cacoullos & Bablanian, 1991).

Viability of the cell is controlled by many different and distinct signals. The expression of anti-apoptotic proteins, such as Bcl-2 or Bcl-x_L, can inhibit apoptosis, while expression of pro-apoptotic proteins, such as Bax or Bak, can accelerate cell death. It has been shown that Bcl-2 family proteins form homo- and heterodimers (Oltvai *et al*., 1993) and that the relative levels of the anti- and pro-apoptotic members of the Bcl-2 family appear to be a key determinant of the fate of cells when confronted with an apoptotic stimulus (Korsmeyer, 1999). It is generally accepted that Bcl-2 family proteins exert their effects mainly by controlling mitochondrial permeability transition, but the exact mechanism of their function remains unknown.

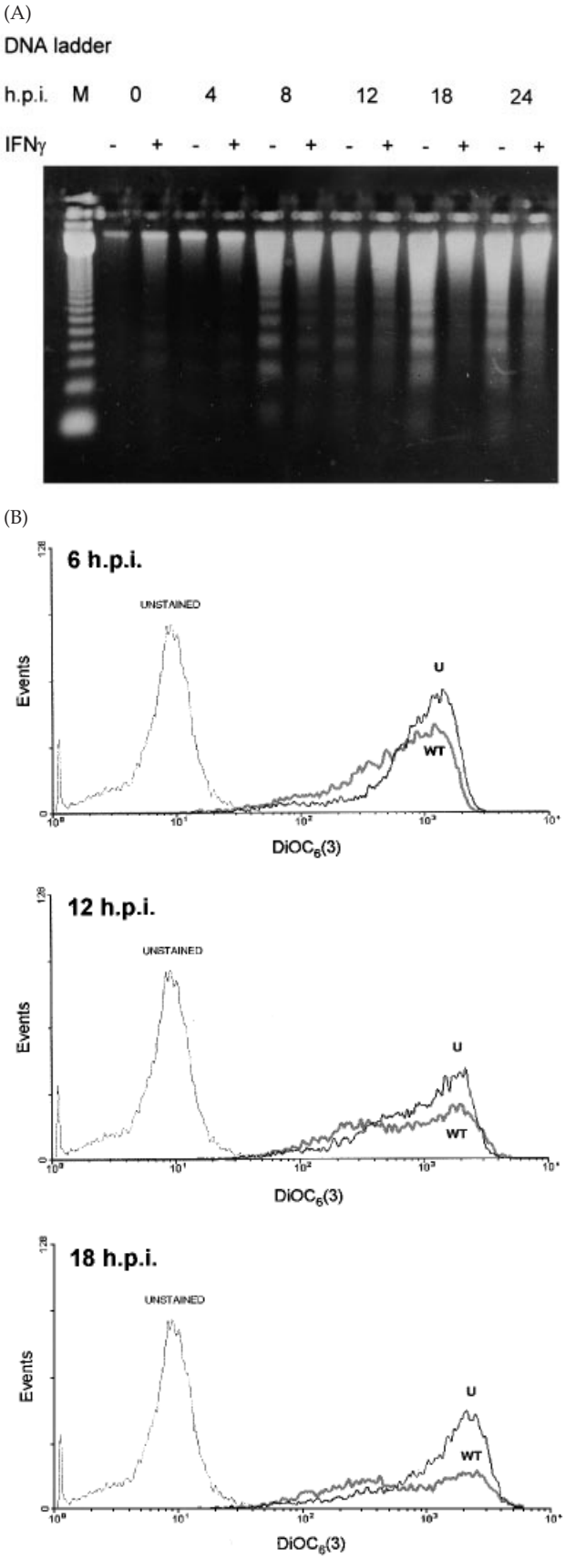
Here, we demonstrate for the first time that cells of a monocyte/macrophage lineage undergo apoptosis when infected with VV. We have observed that NO is not responsible for apoptosis in macrophages infected by VV. However, our results suggest that VV early gene expression triggers the induction phase of apoptosis, while decreased levels of the anti-apoptotic Bcl-x_L mediate the effector phase. The significance of the loss of Bcl-x_L was established by inhibition of VV-induced apoptosis on expression of Bcl-2 using a recombinant VV.

Methods

■ **Chemicals.** All the media and growth supplements were purchased from Gibco BRL or PAA Laboratories. Aminoguanidine, cytosine arabinoside, actinomycin D, cycloheximide and psoralen were purchased from Sigma.

■ **Cells.** The mouse monocyte/macrophage cell line J774.G8 was grown in Dulbecco's modified Eagle's medium (DMEM), with 4·5 g/l glucose, supplemented with 10% heat-inactivated fetal calf serum and antibiotics (1×10^5 U/l penicillin, 100 mg/l streptomycin) (10% FCS DMEM). In some experiments, cells were pretreated for 18 h with mouse recombinant IFN-γ (Genzyme), as specified below. The African green monkey kidney cell line BSC-40 was grown in DMEM supplemented with 10% neonatal calf serum and antibiotics (10% NCS DMEM). The cells were maintained at 37 °C in a 5% CO_2 atmosphere with 95% humidity.

■ **Viruses.** VVs used included wild-type (WT) VV, strain Western Reserve (WR; ATCC VR-119), recombinant VV expressing luciferase under the control of the VV early/late promoter p7.5 (VVLUC; Rodriguez *et al*., 1989) used as a control for other recombinant viruses, and analogous recombinant VVs expressing human proto-oncogene Bcl-2 in sense (VVBcl2+) or antisense (VVBcl2−) orientations. Recombinant viruses with the Bcl-2 gene were prepared by homologous recombination into the thymidine kinase (TK) region of WT VV (Mackett *et al*., 1982) using VV insertion vectors pSC11-Bcl2+ or pSC11-Bcl2− generated and kindly provided by S. B. Lee (Lee, 1994). The insertion and Bcl-2 expression were confirmed by Southern, Northern and Western blot analyses (not shown). Viruses were propagated in BSC-40 cells and purified by sucrose gradient sedimentation (Joklik, 1962). Virus titres



were determined by serial dilutions and plaque assays in BSC-40 cells. For virus infection of macrophages, purified viruses were added at m.o.i.s specified in each experiment and allowed to adsorb to cells for 1 h. After removal of inoculum, cells were supplemented with 10% FCS DMEM. At various times after infection, the cells were collected and subjected to further analysis. For virus growth determination, cells were grown in 24-well plates, with $0{\cdot}5 \times 10^6$ cells in 1 ml in each well, infected at an m.o.i. of 1, and virus titres were determined using 100 μl of the original sample volume of 1 ml. Inactivated viruses were prepared by boiling for 5 min or by treatment with psoralen and UV. For UV-inactivation, viruses (WT $2{\cdot}7 \times 10^8$ p.f.u./ml, VVLUC $3{\cdot}78 \times 10^8$ p.f.u./ml) were suspended in Hanks' balanced salt solution supplemented with 0·1% BSA and psoralen at a final concentration 2 μg/ml. The virus (0·5 ml) was incubated in a 35 mm well at room temperature for 10 min and then irradiated for 5 min with 365 nm UV (Ramsey-Ewing & Moss, 1998). The titres of inactivated viruses were determined on a BSC-40 cell monolayer. After this treatment, no plaque-forming activity was found.

■ **Treatment with inhibitors.** iNOS inhibitor, aminoguanidine (AG; 1 mM), cytosine arabinoside (AraC; 44 μg/ml), actinomycin D (AcD; 5 μg/ml) or cycloheximide (CHX; 100 μg/ml) were present during virus inoculation as well as at later times after infection.

■ **Flow cytometry.** Cell viability and apoptosis were determined by flow cytometry using a FACScan (Becton Dickinson) equipped with the software Lysis II or Cell Quest. Macrophages were vitally stained for 15 min with the potentiometric dyes 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$], rhodamine 123 (Rh 123), or tetramethylrhodamine methyl ester (TMRM) at final concentrations of 20 nM, 80 nM and 100 nM, respectively; propidium iodide (PI; final concentration 2 μg/ml) was then added shortly before measurement to distinguish between live and dead cells. The specificity of fluorescence of the potentiometric dyes was confirmed by inhibition of their accumulation by an uncoupler of the respiratory chain, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP). A $DiOC_6(3)$-negative or low positive and PI-negative population was considered as apoptotic, a $DiOC_6(3)$-positive and PI-negative population was considered as live cells, and a $DiOC_6(3)$-negative and PI-positive population represented necrotic or late apoptotic cells. The number of apoptotic cells was expressed as a percentage of $DiOC_6(3)$-negative or low positive events in a PI-negative population. Apoptotic cells were also detected using the Annexin-V-FLUOS Staining kit (Roche Diagnostics). The percentage of infected macrophages was determined using FITC-labelled rabbit polyclonal IgG against wild-type VV, strain WR (Seva-Imuno Praha, Czech Republic). Macrophages were collected, washed with PBS and stained with FITC-labelled rabbit polyclonal IgG (dilution 1:500). Cells were incubated for 30 min on ice, washed with PBS, stained with PI and processed by FACS analysis.

■ **DNA ladder.** Low molecular mass DNA was isolated as previously described (Lee & Esteban, 1994). Briefly, at the indicated times after

Fig. 1. (A) VV induces apoptosis of infected macrophages. J774.G8 cells ($1{\cdot}5 \times 10^6$), untreated or pretreated for 18 h with 25 U/ml of IFN-γ, were infected with 5 p.f.u./cell of wild-type (WT) VV and grown in six-well plates. At the indicated times after infection, cells were collected and low molecular mass DNA was isolated and resolved by 2% agarose gel electrophoresis. (B) VV induces a decrease in mitochondrial membrane potential ($\Delta\Psi_m$). J774.G8 cells ($0{\cdot}5 \times 10^6$) were infected with 1 p.f.u./cell of WT VV and grown in 24-well plates. At the indicated times after infection, cells were collected, stained with $DiOC_6(3)$ and PI, and analysed by flow cytometry. Graphs represent results of FACS analysis expressed as histograms of $DiOC_6(3)$ fluorescence in the PI-negative population. Representative results of one of three experiments are shown. −, no IFN-γ pretreatment; +, pretreatment with IFN-γ; M, DNA molecular mass marker (123 bp ladder); U, uninfected cells; WT, infected cells.

infection, the cells were collected in medium, lysed in buffer containing 20 mM Tris, 10 mM EDTA and 1% Triton X-100, and high molecular mass DNA was removed by centrifugation at 10000 ***g*** for 10 min. Supernatant containing low molecular mass DNA was extracted with phenol–chloroform–isoamyl alcohol (25:24:1), and low molecular mass DNA was precipitated with ethanol, resuspended in 10 mM Tris–HCl (pH 8·1), 1 mM EDTA, and treated with 5 μg/ml of RNase A at 37 °C for 1 h. DNA was resolved by 2% agarose gel electrophoresis in 1 × TBE and visualized by UV after ethidium bromide staining.

■ **Western blot analysis.** SDS–PAGE and Western blot analysis were performed as previously described (Laemmli, 1970; Harlow & Lane, 1988), using enhanced chemiluminescence (ECL). Bcl-2, Bcl-x_L and Bax were detected with rabbit polyclonal antibodies specific for the individual proteins (dilution 1:500; Santa Cruz Biotechnology); peroxidase-conjugated goat anti-rabbit IgG was used as a secondary antibody (dilution 1:5000; Cappel Research Products). *β*-Actin was detected with goat polyclonal antibody (dilution 1:100; Santa Cruz Biotechnology) and peroxidase-conjugated donkey anti-goat IgG as a secondary antibody (dilution 1:5000; Jackson ImmunoResearch Laboratories). Densitometric analysis of the Western blots was performed using image analysis software Lucia 4.6 (Laboratory Imaging Ltd).

■ **NO determination.** Production of NO was characterized by measuring the accumulation of nitrite, a NO oxidation product. Nitrite was determined in the culture medium by a diazotation assay with Griess reagent (Griess, 1879; Bogle *et al*., 1992). Briefly, a 100 μl aliquot of each sample was mixed with an equal volume of Griess reagent (0·5% sulfanilamide, 0·05% naphthylethylenediamine and 2·5% H_3PO_4), and absorbance at 550 nm was determined after a 10–15 min incubation at room temperature. Sodium nitrite was used as a standard.

Results

VV induces apoptosis of infected macrophages

VV infects a broad range of host cells, usually causing their lysis (i.e. necrosis). In cultured cells, VV replication is mostly insensitive to IFN-*α*/*β* (Paez & Esteban, 1984), but it is inhibited by IFN-*γ* in mouse peritoneal, thioglycolate-elicited macrophages as well as in macrophage cell lines (Karupiah *et al*., 1993; Mělková & Esteban, 1994). Despite the inhibition of VV replication by IFN-*γ*, we have observed that both the untreated and IFN-*γ*-activated mouse macrophage cell line J774.G8 underwent apoptosis on infection by VV (Mělková, 1995). As shown in Fig. 1A, apoptosis was first characterized by a typical DNA fragmentation in agarose gel electrophoresis. Both untreated and IFN-*γ*-activated macrophages revealed typical DNA fragmentation around 8 h post-infection (p.i.). However, DNA fragmentation was more pronounced in untreated cells.

In most of the experiments, an m.o.i. of 1, which typically renders about 30% of cells infected, was used to avoid rapid damage of cells and to make the conditions more similar to the physiological state. Under the experimental conditions used, VV yields were determined to be typically five to ten times lower in IFN-*γ*-pretreated macrophages than in untreated ones (representative results for cells grown in a 24-well plate, $0{\cdot}5 \times 10^6$ cells in 1 ml in each well, m.o.i. of 1; titre after removal of inoculum after 1 h of adsorption: untreated cells – $5{\cdot}43 \times 10^4$ p.f.u./ml, IFN-*γ*-pretreated cells – $5{\cdot}04 \times 10^4$ p.f.u./ml; yields at 24 h p.i.: untreated cells – $5{\cdot}85 \times 10^6$ p.f.u./ml, IFN-*γ*-pretreated cells – $1{\cdot}4 \times 10^6$ p.f.u./ml).

VV induces a decrease in mitochondrial membrane potential ($\Delta\Psi_m$) of infected macrophages

DNA fragmentation by endonucleases is a typical but rather late sign of apoptosis. In contrast, changes in mitochondrial membrane potential, $\Delta\Psi_m$, are considered as early events in the effector phase of apoptosis although they reveal lower specificity. $\Delta\Psi_m$ was estimated using flow cytometry analysis after vital staining of the cells with the cationic lipophilic fluorochromes Rh 123, $DiOC_6(3)$ and TMRM. These potentiometric dyes distribute to the mitochondrial matrix as a function of the Nernst equation, thus correlating with $\Delta\Psi_m$ (Darzynkiewicz *et al*., 1982). Cells undergoing apoptosis typically reveal a reduction in incorporation of $\Delta\Psi_m$-sensitive dyes; cellular membrane remains intact (PI-impermeable, reflected as a PI-negative population in flow cytometry) in early phases of apoptosis, while it becomes permeable (PI-positive) during secondary necrosis in later phases. Therefore, we characterized early stages of apoptosis of VV-infected macrophages by changes of $\Delta\Psi_m$ using $DiOC_6(3)$ (Figs 1B and 2A), Rh 123 and TMRM (data not shown). The decrease in $\Delta\Psi_m$ could be observed at 6 h p.i. and became more apparent at 12 and 18 h p.i. (Fig. 1B). Fig. 2(A) represents the result of a dot plot analysis of cells stained with $DiOC_6(3)$ and PI. The percentage of apoptotic cells ($DiOC_6(3)$-negative or low positive and PI-negative population) increases in a time-dependent manner in both WT VV-infected and VVLUC-infected cells, the latter being used as a control for other recombinant viruses. Similar results were observed using Rh123 or TMRM. The percentage of VV-infected macrophages determined using FITC-labelled rabbit polyclonal antibody against VV and flow cytometry (Fig. 2B) was compared with the percentage of apoptotic cells (Fig. 2A; both characteristics determined in a live, PI-negative population), and at individual intervals p.i. more cells were found to be infected than apoptotic. This result is in agreement with the hypothesis that VV infection of the individual cells precedes the decrease in their $\Delta\Psi_m$ and apoptosis.

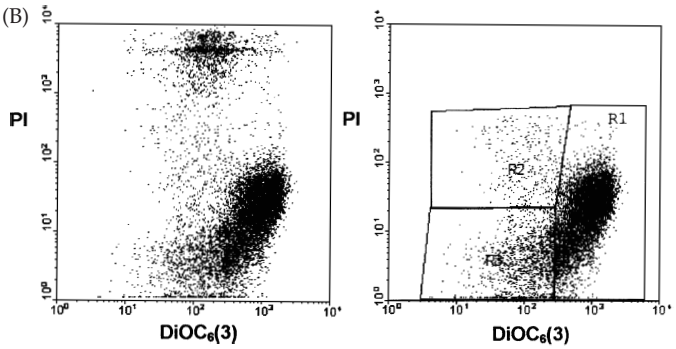
iNOS inhibitor does not prevent apoptosis

NO has been shown to induce apoptosis of IFN-*γ*- and LPS-activated macrophages (Sarih *et al*., 1993). NO also inhibits VV growth in IFN-*γ*-activated macrophages (Karupiah *et al*., 1993). Therefore, we were interested in whether NO could also be responsible for apoptosis of macrophages infected with VV. In infected, untreated cells no increase in nitrite levels compared with uninfected, untreated cells was detected. Aminoguanidine (AG), a relatively specific inhibitor of iNOS (Corbett *et al*., 1992), inhibited NO synthesis by IFN-*γ*-activated macrophages to the level comparable with uninfected, untreated cells

(A)

	Apoptosis (%)			
	0 h p.i. (*n*=3)	6 h p.i. (*n*=3)	12 h p.i. (*n*=4)	18 h p.i. (*n*=4)
U	6·90±0·67	5·66±0·89	8·56±1·92	8·28±0·81
WT	–	8·95±2·61	19·29±3·24	24·75±3·41
LUC	–	7·48±1·92	21·02±4·55	26·35±1·77
Bcl-2+	–	5·82±1·58	7·48±1·52	11·48±1·36
Bcl-2–	–	7·62±2·57	16·74±3·15	24·59±2·47

(B)



(C)

Infected cells (%)			
0 h p.i.	6 h p.i.	12 h p.i.	18 h p.i.
–	12·47	19·28	67·07

Fig. 2. (A) Percentage of apoptosis in VV-infected macrophages. J774.G8 cells ($0{\cdot}5 \times 10^6$) were infected with 1 p.f.u./cell of WT VV or with 1 p.f.u./cell of various VV recombinants and grown in 24-well plates. At the indicated times after infection, cells were collected, stained with $DiOC_6(3)$ and PI, and analysed by flow cytometry. The table represents the percentage of apoptotic cells [characterized as $DiOC_6(3)$-negative or low positive] in a PI-negative population at the indicated times after infection. Data represent means of three or four experiments ± standard errors of mean (SEM). (B) The dot plots show all the cells measured (left) and the analysed PI-negative population (right). Region 1 (R1) represents live cells, region 2 (R2) represents apoptotic cells and region 3 (R3) represents cellular debris. (C) Percentage of VV-infected cells. J774.G8 cells ($0{\cdot}5 \times 10^6$) were infected with 1 p.f.u./cell of WT VV and grown in 24-well plates. At the indicated times after infection, cells were collected, stained with FITC-labelled rabbit IgG against VV and PI, and analysed by flow cytometry. The table represents the percentage of infected cells, detected by FITC-labelled rabbit IgG against VV in a PI-negative population. Representative results of one of two experiments are shown. U, Uninfected cells; WT, cells infected with WT VV; LUC, cells infected with control VV expressing luciferase; Bcl-2+ or Bcl-2−, cells infected with VV expressing Bcl-2 in sense or antisense orientations, respectively.

(characterized by the levels of nitrite in culture medium). In these experiments, AG rescued VV growth inhibited by IFN-γ (data not shown). However, AG was not able to prevent apoptosis of VV-infected, IFN-γ-activated macrophages or untreated macrophages, characterized by flow cytometry (Table 1) as well as by DNA ladder results (data not shown). Since the inhibitor of iNOS could not prevent apoptosis of VV-infected macrophages, we concluded that NO is not a key mediator in VV-induced apoptosis in macrophages.

VV virion antigens or late genes are not responsible for induction of apoptosis in macrophages

In searching for the mechanism by which VV might induce apoptosis of infected macrophages, we wanted to determine whether apoptosis was induced actively by some VV gene product or whether VV served only as an antigenic stimulus to trigger macrophage apoptosis. In these experiments we treated macrophages with heat-inactivated VV or with a live VV. The percentage of apoptotic cells remained low in the macrophages treated with heat-inactivated VV, while macrophages infected with live VV revealed increasing apoptosis. Similar results were obtained in both untreated and IFN-γ-activated macrophages (data not shown). As an additional control, psoralen/UV-inactivated VV was used; this virus was replication-incompetent and also did not cause any apoptosis. These results suggested that apoptosis was actively induced by VV gene expression and not by an antigenic stimulus.

VV replication is a cascade event, completion of each step being a pre-requisite for the following one (Moss, 1990). In order to distinguish which VV gene products might induce apoptosis, we treated macrophages with AraC to inhibit VV DNA synthesis and, consequently, expression of VV late

Table 1. iNOS inhibitor does not prevent apoptosis

J774.G8 cells ($0{\cdot}5 \times 10^6$), untreated or pretreated for 18 h with 25 U/ml of IFN-γ, were infected with 1 p.f.u./cell of VV expressing luciferase (VVLUC) and grown in 24-well plates in the absence or presence of 1 mM aminoguanidine (AG). At the indicated times after infection, cells were collected, stained with $DiOC_6(3)$ and PI, and analysed by flow cytometry. The table represents the percentage of all apoptotic events [$DiOC_6(3)$-negative or low positive] in a whole PI-negative population at the indicated times after infection. Representative results of one of three experiments are shown. U, Uninfected cells; LUC, cells infected with VVLUC; AG, aminoguanidine.

	Apoptosis (%)				
	0 h p.i.	6 h p.i.	12 h p.i.	18 h p.i.	24 h p.i.
Untreated cells					
U/0	10·49	8·05	6·98	12·94	8·15
U/AG	—	7·06	5·73	8·41	8·51
LUC/0	—	10·45	17·47	25·09	32·62
LUC/0/AG	—	10·32	14·36	22·85	30·79
IFN-γ-treated cells					
U/IFN-γ	9·71	9·92	10·16	10·86	10·75
U/IFN-γ/AG	—	10·24	7·45	9·26	6·68
LUC/IFN-γ	—	13·76	17·04	37·87	42·44
LUC/IFN-γ/AG	—	14·91	15·89	37·79	48·99

Table 2. VV-induced apoptosis is not mediated by VV late proteins: inhibition of RNA and protein synthesis mimics the effects of VV infection

J774.G8 cells ($0{\cdot}5 \times 10^6$) were infected with 1 p.f.u./cell of VVLUC and treated with AraC (44 μg/ml), AcD (5 μg/ml) or CHX (100 μg/ml); cells were grown in 24-well plates. At the indicated times after infection, cells were collected, stained with $DiOC_6(3)$ and PI, and analysed by flow cytometry. The table represents the percentage of all apoptotic events [$DiOC_6(3)$-negative or low positive] in a whole PI-negative population at the indicated times after infection. Representative results of one of two experiments are shown. U, Uninfected cells; LUC, cells infected with VVLUC.

	Apoptosis (%)			
	0 h p.i.	6 h p.i.	12 h p.i.	18 h p.i.
Uninfected cells				
U	0·79	3·70	7·04	3·74
U/AraC	—	2·49	5·35	6·09
U/AcD	—	5·07	18·46	29·25
U/CHX	—	4·58	19·50	26·58
Infected cells				
LUC	—	2·61	5·97	16·37
LUC/AraC	—	2·35	8·01	18·93
LUC/AcD	—	2·89	21·29	32·20
LUC/CHX	—	4·82	24·20	31·67

genes. Table 2 represents a flow cytometric analysis of the effects of AraC on macrophage apoptosis. In the uninfected cells, AraC did not affect the background, low percentage of apoptosis; however, in cells infected in the presence of AraC, apoptosis occurred in a similar percentage to untreated infected cells. Therefore, apoptosis in VV-infected macrophages is an early event in virus infection.

Consequently, we used inhibitors of RNA and protein synthesis (AcD and CHX, respectively) to block expression of VV early genes. However, concentrations of AcD and CHX

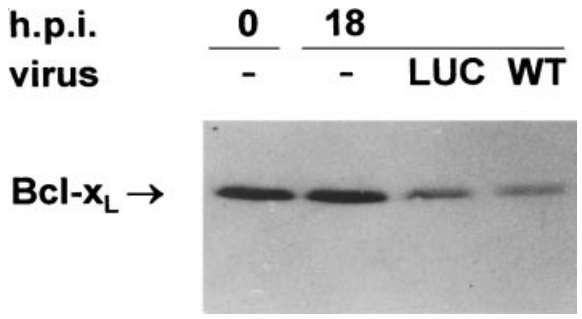


Fig. 3. VV induces a decrease of Bcl-x_L levels. J774.G8 cells (3×10^6) were infected with 1 p.f.u./cell of WT VV or VV expressing luciferase (VVLUC) and grown in 60 mm plates. At 18 h p.i., cells were collected, lysed in Laemmli reducing sample buffer and resolved by 14% SDS–PAGE. Bcl-x_L was detected by a Western blot analysis using rabbit polyclonal antibody against Bcl-x_L and enhanced chemiluminescence. WT, Cells infected with WT VV; LUC, cells infected with VVLUC.

commonly used to inhibit VV early gene expression (Moss, 1990) induced apoptosis of uninfected cells, and a simultaneous infection by VV did not further increase the percentage of apoptosis (Table 2; an m.o.i. of 1 used throughout the experiments does not render all cells infected). Thus, the effect of a pharmacological inhibition of RNA and protein synthesis in the uninfected cells is similar to the effect of VV infection of the cells, and induction of apoptosis by VV might be connected with VV early gene expression and VV-mediated inhibition of host-cell macromolecular synthesis.

VV induces a marked decrease of the anti-apoptotic Bcl-x_L and a limited increase of the pro-apoptotic Bax

Members of the Bcl-2 family regulate the process of apoptosis at the level of mitochondria, and changes in their

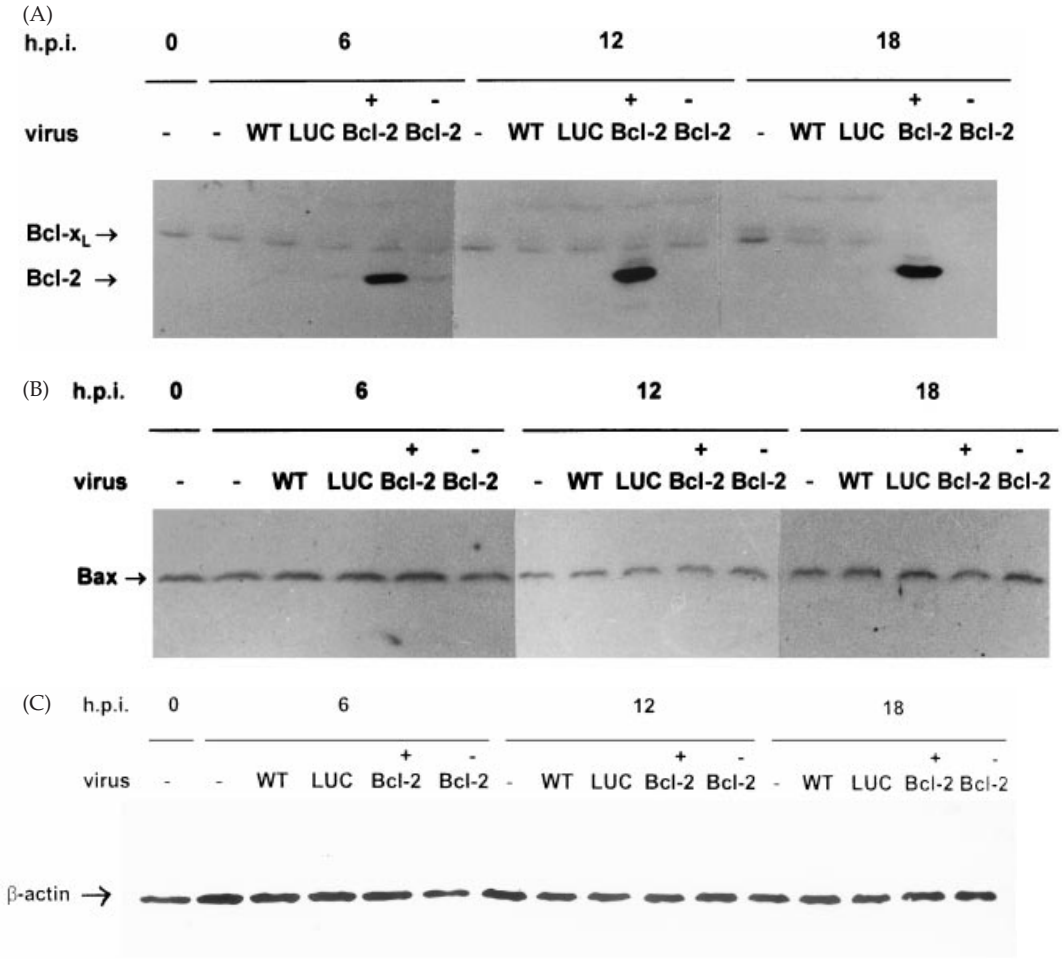


Fig. 4. Changes in the endogenous Bcl-x_L and Bax protein levels following VV infection. J774.G8 cells (3×10^6) were infected with 1 p.f.u./cell of WT VV or VVs expressing either luciferase (VVLUC) or Bcl-2 in sense or antisense orientations (VVBcl2+ or VVBcl2−), and grown in 60 mm plates. At the indicated times after infection, cells were collected, lysed in Laemmli reducing sample buffer and resolved by 14% SDS–PAGE. Bcl-x_L, Bcl-2 (A), Bax (B), and β-actin (C) were detected by Western blot analysis using polyclonal antibodies against individual proteins and enhanced chemiluminescence. WT, Cells infected with WT VV; LUC, cells infected with VVLUC; Bcl-2+ or Bcl-2−, cells infected with VV expressing Bcl-2 in sense or antisense orientations, respectively.

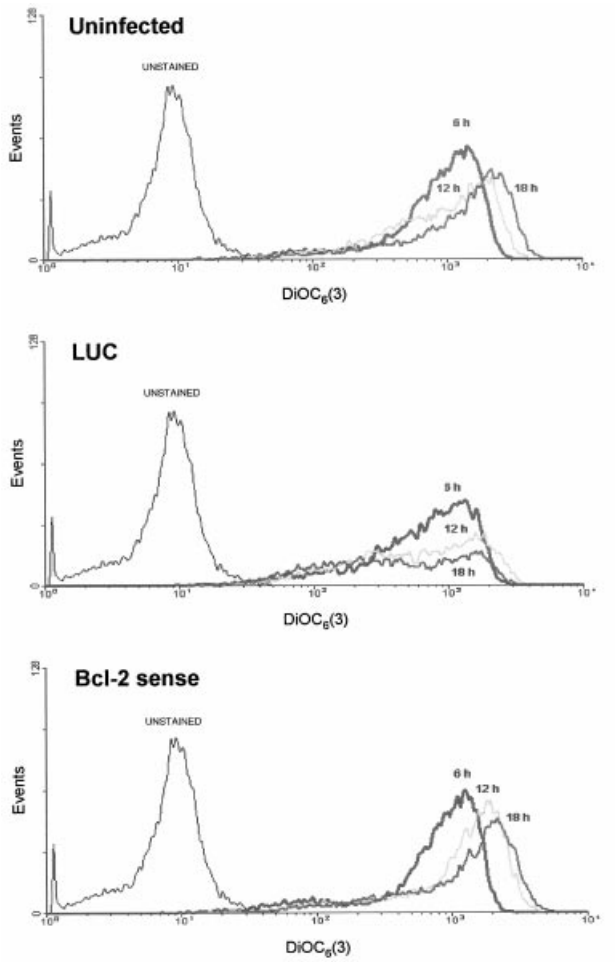


Fig. 5. Expression of Bcl-2 by VV can rescue the decrease of mitochondrial membrane potential ($\Delta\Psi_m$). J774.G8 cells ($0{\cdot}5 \times 10^6$) were infected with 1 p.f.u./cell of VV expressing either luciferase (VVLUC) or Bcl-2 in the sense orientation (VVBcl2+), and grown in 24-well plates. At the indicated times after infection, cells were collected, stained with $DiOC_6(3)$ and PI, and analysed by flow cytometry. Graphs represent results of FACS analysis expressed as histograms of $DiOC_6(3)$ fluorescence in the PI-negative population. Representative results of one of three experiments are shown.

relative levels are known to promote cell survival or induce cell death. Therefore, using Western blot analysis, we first determined the effect of VV infection on levels of the major anti-apoptotic proteins, Bcl-2 and Bcl-x_L. While we did not detect any endogenous Bcl-2 protein, even in uninfected cells, Bcl-x_L levels were found to be decreased at 18 h p.i. in cells infected by both WT VV or another control virus, VVLUC (Fig. 3). When performing a time-course experiment using WT VV as well as various VV recombinants, the levels of Bcl-x_L were found to decrease in a time-dependent manner relative to uninfected controls (Fig. 4A). In contrast, levels of pro-apoptotic Bax were found somewhat increased in most of the infected samples when compared with the appropriate uninfected controls (Fig. 4B). Fig. 4(C) shows relatively comparable levels of the house-keeping β-actin in the infected and uninfected samples. Densitometric analysis of the Western blots revealed a fourfold decrease of Bcl-x_L and a 1·7-fold increase of Bax proteins at 18 h p.i. (mean of three independent experiments). Standardization of the relative densities of Bcl-x_L or Bax to the relative densities of β-actin in each individual sample also revealed decreasing or increasing tendencies, respectively (not shown).

Table 3. Bcl-2 expression prevents annexin-V positivity

J774.G8 cells ($0{\cdot}5 \times 10^6$) were infected with 1 p.f.u./cell of WT VV or VV expressing either luciferase (VVLUC) or Bcl-2 in sense or antisense orientations (VVBcl2+ or VVBcl2−), and grown in 24-well plates. At 18 h p.i., cells were collected, stained with the Annexin-V-FLUOS kit and analysed by flow cytometry. The table represents the percentage of apoptotic cells (Annexin-V-positive) in a PI-negative population. Representative results of one of two experiments are shown. Total, % of all apoptotic events in the whole PI-negative population; Net, % of apoptotic events in the uninfected sample subtracted from infected samples as background; U, uninfected cells; WT, cells infected with WT VV; LUC, cells infected with VVLUC; Bcl-2+ or Bcl-2−, cells infected with VV expressing Bcl-2 in sense or antisense orientations, respectively.

	Apoptosis (%)		
	0 h p.i.	18 h p.i.	
	Total	Total	Net
U	3.47	8.77	0.00
WT	—	45.92	37.15
LUC	—	44.87	36.10
Bcl2+	—	23.18	14.41
Bcl2−	—	56·70	47·93

Expression of Bcl-2 by VV can substitute for the loss of Bcl-x_L

Finally, in order to prove that decreased levels of Bcl-x_L were involved in apoptosis of VV-infected macrophages, we substituted for the loss of Bcl-x_L by expressing its functional homologue Bcl-2. As revealed by preservation of $\Delta\Psi_m$ (Fig. 5), exposure of phosphatidyl serine on the cell surface (annexin-V positivity; Table 3), as well as by cellular morphology (data not shown), expression of Bcl-2 by a VV recombinant rescued the infected macrophages from apoptosis. In the same time interval, Bcl-2 expression did not affect VV growth. The expression of Bcl-2 by a VV recombinant was confirmed by Western blot analysis (Fig. 4A). In contrast, macrophages infected by WT VV or by control VV recombinants expressing various other genes underwent apoptosis. As expected, expression of Bcl-2 antisense mRNA did not further affect the course of apoptosis (Fig. 2A).

Discussion

Macrophages play a critical role in connecting innate and specific immune responses. Natuk & Holowczak (1985) reported that VV DNA replication and growth are inhibited in freshly isolated mouse peritoneal macrophages. Macrophage apoptosis due to VV infection might lead to a deficiency of certain cytokines normally produced by macrophages (e.g. IFN-α or interleukins), and thus alter an immune response mounted by certain cell types, namely T cells. Indeed, exogenous administration of IFN-α/β significantly reduced VV growth *in vivo*, as characterized by expression of only VV early proteins in mouse peritoneal cells and spleen (Rodriguez *et al.*, 1991). Since VV is known to be resistant to IFN-α/β *in vitro* (Paez & Esteban, 1984; Chang *et al.*, 1992; Davies *et al.*, 1992; Rivas *et al.*, 1998), this result suggests that the administered IFN-α/β did not directly inhibit VV growth in IFN-α/β-responsive cells. Instead, IFN-α/β might have substituted for a cytokine deficiency caused by VV (e.g. due to a VV-induced macrophage apoptosis) and led to activation of and/or cytokine production by other immune cells that would inhibit VV growth by a distinct mechanism. Such a mechanism might involve IFN-γ-mediated induction of iNOS or a CTL response (van den Broek *et al.*, 1995). Likewise, VV was recently described to induce apoptosis of dendritic cells, thus preventing their maturation and inhibiting their function as antigen-presenting cells and, consequently, T cell activation (Engelmayer *et al.*, 1999).

Our results suggest that apoptosis of VV-infected macrophages is mediated by VV early gene products. It occurs in the absence of VV DNA synthesis and/or expression of VV late genes in IFN-γ-pretreated macrophages or in the presence of AraC. It has been previously shown that apoptosis of HeLa cells infected with VV lacking the E3L gene was prevented on inhibition of VV DNA synthesis and late genes expression by AraC; this apoptosis was, however, due to activation of IFN-induced dsRNA-activated protein kinase (PKR) by dsRNA accumulated during expression of VV late genes (Colby *et al.*, 1971; Kibler *et al.*, 1997). In contrast, WT VV as well as the recombinant VVs used in this study encode the early genes E3L and K3L, the products of which counteract IFN-inducible dsRNA-activated PKR and 2′-5′ oligoadenylate synthetase (2-5A synthetase)/RNaseL system (Chang *et al.*, 1992; Davies *et al.*, 1992; Rivas *et al.*, 1998). Therefore, it is not clear if these systems could be involved in the apoptosis of VV-infected macrophages described here, despite the fact that both the 2-5A synthetase/RNaseL system and PKR are able to trigger apoptosis (Lee & Esteban, 1994; Diaz-Guerra *et al.*, 1997; Zhou *et al.*, 1997). Possibly, high endogenous levels of the dsRNA-activated 2-5A synthetase/RNaseL system or PKR in macrophages might overcome the VV-encoded early proteins E3L and K3L that should normally counteract them.

Treatment of macrophages with the RNA or protein synthesis inhibitors, AcD or CHX, respectively, induced apoptosis of uninfected macrophages. Infection by VV in the presence of these inhibitors did not further increase the percentages of apoptotic cells, possibly suggesting that an additional VV-induced apoptosis was prevented by the lack of expression of VV early genes. Also, AcD- and CHX-mediated inhibition of protein synthesis might mimic VV-mediated shut-off of host-cell protein synthesis (Kit & Dubbs, 1962; Shatkin, 1963; Becker & Joklik, 1964). Since the shut-off is mediated by VV early gene products or by VV virion proteins (Buendia *et al.*, 1987; Bablanian *et al.*, 1993), such a mechanism would be compatible with apoptosis induced in infected macrophages. VV-induced apoptosis was shown to be mediated by VV early gene products or by virion proteins in other systems as well. In VV-induced apoptosis of the immature B-lymphocyte cell line, WEHI-231, several VV-specific proteins were expressed, but no viral DNA synthesis or virus progeny were detected (Baixeras *et al.*, 1998). In contrast, in CHO cells, apoptosis was induced after binding of VV to the cell without any requirement for virus gene expression (Ramsey-Ewing & Moss, 1998). Our results with heat- or UV-inactivated VV suggest that VV early gene expression is necessary for apoptosis. However, a VV deletion mutant deficient, for example, in virion-associated protein kinase (Buendia *et al.*, 1987) would better clarify this point.

We have previously demonstrated that VV inhibited host-cell protein synthesis in J774.G8 macrophages (Mělková & Esteban, 1994), and in this study, inhibition of RNA and protein synthesis by AcD and CHX, respectively, mimicked the induction of apoptosis by VV. Therefore, we assumed that levels of certain proteins critical for the control of apoptosis could be affected. Specifically, we have focused on the levels of the proto-oncogenes Bcl-2, Bcl-x_L and Bax, the ratios of which are considered to control the effector phase of apoptosis (Kroemer, 1997; Korsmeyer, 1999). We were not able to detect any endogenous levels of Bcl-2 in J774.G8 macrophages; instead, Bcl-x_L was present, and infection with VV induced a decrease in its level, while levels of the pro-apoptotic homologue Bax were increased in most of the infected samples (results of three independent experiments). Induction of expression of Bax simultaneously with down-regulation of Bcl-2 in response to various apoptotic stimuli has been reported previously (Gillardon *et al.*, 1995). Additionally, Bcl-x_L protein could be preferentially degraded on infection with VV in a way similar to glutathione depletion-induced degradation of Bcl-2 and apoptosis in cholangiocytes (Celli *et al.*, 1998).

We demonstrated the role Bcl-x_L plays in VV-induced macrophage apoptosis by substituting for its function with expression of its homologue, Bcl-2, using a recombinant VV. Bcl-2 preserved mitochondrial membrane potential, prevented exposure of phosphatidyl serine on the cell surface and preserved normal cellular morphology despite relatively increased levels of Bax. The exact mode of action of Bcl-2 homologues still remains elusive, but most hypotheses stress their effects on mitochondrial function (Kroemer, 1997; Reed *et*

al., 1998). Since VV growth was comparable in the presence as well as in the absence of Bcl-2 expression, it seems unlikely that Bcl-2 could fundamentally change the properties of VV-expressed proteins or the VV growth cycle. It might rather be predicted that Bcl-2 could modify the metabolic consequences imposed on the host cell by VV growth. For example, Bcl-2 might prevent changes of the intracellular milieu induced by VV or could affect the extent of VV-mediated shut-off of host-cell macromolecular synthesis. These and other questions remain to be explored.

In conclusion, we have demonstrated for the first time that VV induces apoptosis of infected macrophages. We did not find any role of NO in this VV-induced apoptosis. However, VV induced a marked decrease of cellular levels of Bcl-x_L simultaneously with a limited increase in levels of Bax. The importance of the loss of Bcl-x_L was demonstrated by prevention of apoptosis on expression of Bcl-2, a functional homologue of Bcl-x_L.

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